Transport of calcium across caco-2 cells in the presence of inositol hexakisphosphate

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Abstract

The Caco-2 enterocyte cell model was used to determine whether low concentrations of inositol hexakisphosphate ($InsP_6$, phytate) may enhance calcium absorption, as was recently reported for several nonintestinal types of mammalian cells. Three-week-old Caco-2 cell layers grown in tissue culture plates with or without cell culture inserts were incubated with $[^{45}Ca]Cl_2$ to examine calcium transport or uptake, respectively. No increases in calcium uptake were observed in the presence of micromolar levels of $InsP_6$, whereas millimolar levels of $InsP_6$ gave incongruous results depending on the calcium concentration. The fractional apical-to-basolateral calcium transport was significantly inhibited ($P < .05$) by $InsP_6$ levels of 100 μmol/L and above, but the total molar amount of calcium transported still increased with increasing concentrations of calcium. It was concluded that $InsP_6$ cannot enhance calcium absorption by Caco-2 cells, Caco-2 cell transport is a better indicator of calcium availability than uptake, and higher consumption of calcium may overcome the inhibition of $InsP_6$.

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1. Introduction

Dietary *myo*-inositol hexakisphosphate ($InsP_6$, phytate), which comprises approximately 0.5% to 2% of the dry weight of mature seeds, forms insoluble complexes with minerals and reduces their absorption from the gut [1]. For humans, the major concern has been the frequently observed reduction in bioavailability of minerals such as calcium, iron, zinc, and magnesium. In agriculture, a growing problem is the environmental impact of farm animal wastes containing undigested $InsP_6$, which can pollute surface water and spur the overgrowth of various microorganisms. The nutritional properties of phytate have most often been studied in vitro or in live animals. The Caco-2 cell model also has been used to examine the effects of $InsP_6$ and other inositol phosphates on the absorption of iron [2-6], zinc [2,7], and calcium [7,8]. Confluent Caco-2 (colon adenocarcinoma) cells spontaneously differentiate in culture to form a polarized epithelium that can be used to study the intestinal absorption of micronutrients and drugs [9].

When foods or feeds are consumed, phytases derived from dietary components, supplements, intestinal cells, or intestinal microflora may degrade the $InsP_6$ to a spectrum of inositol phosphates and inorganic phosphate [1]. Some inositol phosphates, including $InsP_6$, have biologic activities in addition to the formation of insoluble mineral complexes. With varying potency, numerous inositol phosphates can bind to inositol 1,4,5-trisphosphate receptors and signal the release of calcium from intracellular stores or the uptake of calcium from outside the cell [10]. Intracellular $InsP_6$ at micromolar levels has been shown to promote calcium influx through calcium channels in the plasma membranes of pancreatic β cells [11], hippocampal neurons [12], and vascular smooth muscle cells [13]. It is believed that $InsP_6$

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increases calcium channel activity by acting as a general intracellular signaling molecule operating through multiple mechanisms, including inhibition of protein phosphatases and stimulation of adenyl cyclase [14].

The physiologic concentrations of $InsP_6$ and calcium in various rat tissues are approximately 0.6 to 6 μmol/L and 150 to 850 μmol/L, respectively [15]. There is evidence that most of the extracellular $InsP_6$ in mammalian tissues may come from the absorption of dietary $InsP_6$, whereas intracellular $InsP_6$ is made inside the cells [16]. If some of the $InsP_6$ in foods or feeds can be absorbed intact, it might enhance the absorption of calcium. The purpose of the following work was to test this possibility with Caco-2 cell layers and to determine their usefulness for studying the effects of inositol phosphates on calcium absorption.

2. Experimental procedures

2.1. Materials

Caco-2 cells (American Type Culture Collection, Manassas, Va) between passages 20 and 50 were grown under 5% CO_2 in a 37°C incubator in 25-cm^2 flasks containing 10 mL of MEM Alpha Medium with glutamine (Life Technologies, Rockville, Md), 10% fetal bovine serum (Life Technologies), and 8 mg/mL gentamycin (Sigma Chemical Company, St Louis, Mo). Fresh medium was supplied every 2 or 3 days, and the cells were passaged at 7-day intervals. Falcon tissue culture plates and cell culture inserts were from Becton Dickinson and Company (Franklin Lakes, NJ). *Myo*-inositol hexakisphosphate dodecasodium salt from rice and *N*-ethylmaleimide were obtained from Sigma.

2.2. Calcium uptake

Aliquots of 10^5 cells per milliliter were seeded in 24-well Falcon cell culture plates. Fresh medium was supplied every 2 or 3 days for 22 or 23 days and 24 hours before an experiment. After washing 3 times with calcium- and magnesium-free Hanks' balanced salt solution, cells were incubated with 1 mL of absorption buffer (500 μmol/L $CaCl_2$, 130 mmol/L NaCl, 10 mmol/L KCl, 1 mmol/L $MgSO_4$, 5 mmol/L glucose, and 50 mmol/L HEPES, pH 7.0) containing 1 mCi $[^{45}Ca]Cl_2$ (7.35 mCi/mg) and varying concentrations of $InsP_6 \pm$ additional $CaCl_2 \pm$ 1 mmol/L *N*-ethylmaleimide. After shaking for 60 minutes at 50 rpm and 37°C in a C24 Incubator Shaker (New Brunswick Scientific, Edison, NJ), cells were washed 3 times with ice-cold 150 mmol/L NaCl, 1 mmol/L EDTA, and 10 mmol/L HEPES (pH 7.0). Cells were lysed with 1 mL of 1% sodium dodecyl sulfate, and the radioactivity of the lysate was measured with 5 mL of Scinti-Safe Plus 50% liquid scintillation cocktail (Fisher Scientific, Pittsburgh, Pa).

2.3. Calcium transport

Aliquots of 10^5 cells per 500 μL were seeded in Falcon 1.0-mm pore size inserts in 24-well plates containing 800 μL

of culture medium per well. Fresh medium was supplied every 2 or 3 days for 22 or 23 days and 24 hours before an experiment. After washing 3 times with calcium- and magnesium-free Hanks' Balanced Salt Solution, 800 μL of absorption buffer was added to the wells, and 500 μL of absorption buffer containing 1 mCi $[^{45}Ca]Cl_2$ (7.35 mCi/mg) and varying concentrations of $InsP_6 \pm$ additional $CaCl_2$ was pipetted into each insert. After shaking for 60 minutes at 50 rpm and 37°C, 400 μL was removed from the wells (basolateral chambers), and its radioactivity was measured with 5 mL of Scinti-Safe Plus 50%. Resistance, as an indicator of the permeability of the cell layers, was measured with an epithelial voltohmmeter (World Precision Instruments, Sarasota, Fla) after placing the insert in an Endohm-12 tissue resistance measurement chamber (World Precision Instruments) with 0.7 and 4.0 mL of Hanks' Balanced Salt Solution in the insert and chamber, respectively.

2.4. Statistics

Transepithelial resistance comparison of different inserts was analyzed by unpaired *t* test. Multiple means of transport data were evaluated by 1-way analysis of variance, and significant differences at $P < .05$ were determined by post hoc comparisons using the Bonferroni correction with the free online calculator at www.graphpad.com.

3. Results

Two different aspects of calcium absorption by Caco-2 cells were examined in these experiments. Uptake was used to measure the process of calcium entering the cells at the apical surface and was intended to represent the amount of calcium contained inside of the cells. In contrast, transport included entering, moving through the cell, and exiting at the basolateral surface. Preliminary experiments were used to define the concentrations of calcium and $InsP_6$ that would result in the most clearly shown differences when plotted in graphical form. The most demonstrative combinations and
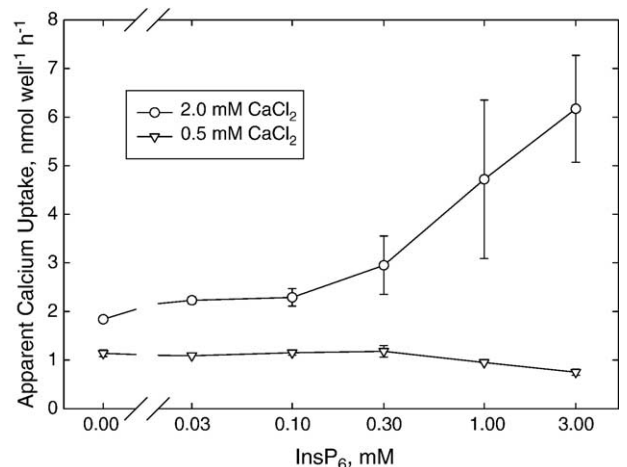

Fig. 1. Effect of $InsP_6$ on the apparent uptake of $[^{45}Ca]Cl_2$ by Caco-2 cells. Error bars represent the ranges of 2 replicates.
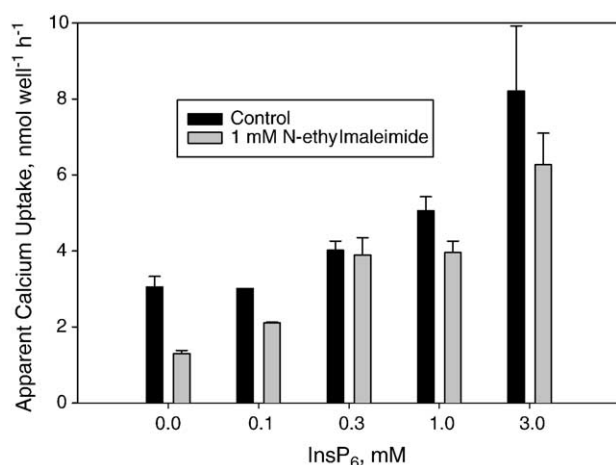
Fig. 2. Effect of *N*-ethylmaleimide on the apparent enhancement of uptake of 2 mmol/L of $CaCl_2$ by Caco-2 cells in the presence of $InsP_6$. Error bars represent the ranges of 2 replicates.
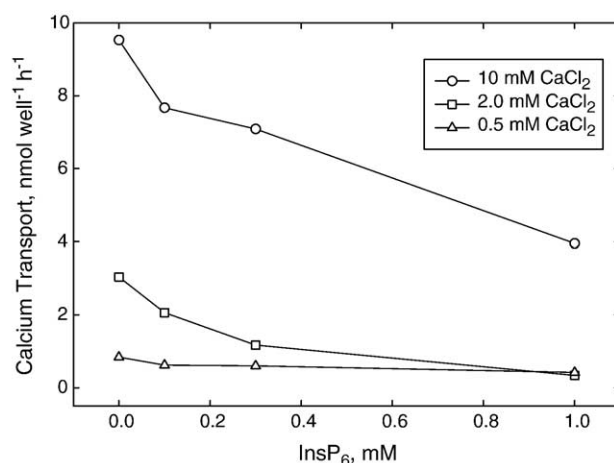

Fig. 3. Effect of $InsP_6$ on the apical to basolateral transport of $[^{45}Ca]Cl_2$ across Caco-2 cell layers. Data represent the means of 3 replicates. Statistics are presented in Table 2, showing the data both as fractional and as molar values.

ranges of concentrations were then used in the following uptake or transport experiments. $InsP_6$ was not measured.

The uptake of calcium ions by Caco-2 cells was investigated at calcium to $InsP_6$ ratios from 0.167 to 66.7. Uptake of 0.5 mmol/L of calcium was inhibited at $InsP_6$ concentrations of 1 mmol/L and above but not at 0.3 mmol/L and below (Fig. 1). The results shown were typical of additional similar experiments, but because only 2 replicates were analyzed, the results were not significant at $P < .05$. In contrast, the uptake of 2 mmol/L of calcium was not affected at $InsP_6$ levels of 0.1 mmol/L and below, but uptake appeared to be enhanced when the $InsP_6$ concentration was 0.3 mmol/L or higher. In an attempt to validate these results, a control experiment was performed using 2 mmol/L of calcium ± 1 mmol/L *N*-ethylmaleimide, a metabolic inhibitor that alkylates cysteine residues in proteins and has been shown to inhibit calcium uptake by Caco-2 cells [17]. As seen in Fig. 2, *N*-ethylmaleimide did not prevent the observed increase in calcium uptake by $InsP_6$. It was concluded that the enhancement was an artifact probably resulting from calcium phytate binding to the surfaces of the cells.

To investigate the effect of $InsP_6$ on calcium transport through Caco-2 cells, a preliminary experiment was conducted to compare the growth of the Caco-2 monolayer on inserts of different pore size and density. Superior values of transepithelial electrical resistance (TEER) were obtained using 1-mm pore inserts compared with 0.4-mm pore high-

density inserts (Table 1). Caco-2 cell layers are generally considered to have an established barrier function when the TEER is at least 200 to 250 Ω cm^2 [8,18]. All 24 replicates of the 1-mm pore inserts gave TEER values above 200, with an average value of 301, compared with an average TEER of only 194 for the 0.4-mm high-density inserts. Accordingly, the 1-mm inserts were used for all subsequent experiments on calcium transport.

The transport of calcium ions through Caco-2 cells was measured at calcium-to-$InsP_6$ ratios from 0.5 to 100. A dose response of transport was observed from 0.5 to 10 mmol/L of calcium, and the inhibition of calcium transport increased from 0.2 to 1.0 mmol/L of $InsP_6$ (Fig. 3). Although the fractional inhibition of calcium transport increased with the concentration of $InsP_6$, the molar calcium transport still increased with the concentration of calcium (Table 2).

Table 1

Permeability of Caco-2 cell layers grown on inserts of different pore size and density

	1-mm pore size	0.4-mm pore size, high density
pore density	1.6×10^6/cm^2	1.0×10^8/cm^2
TEER[a,b]	301 ± 42 Ω cm^2	194 ± 18 Ω cm^2
range of TEER values	210-341 Ω cm^2	166-226 Ωcm^2

[a] Values are means ± SD (n = 24).
[b] Values are significantly different at $P < .0001$.

Table 2

Effect of $InsP_6$ on the fractional and molar $CaCl_2$ transport across Caco-2 cell layers

$CaCl_2$ (mmol/L)	$InsP_6$ (mmol/L)	% Transport	nmol well^{-1} h^{-1}
0.5	0.0	0.34 ± 0.03[a]	0.84 ± 0.07[b]
0.5	0.1	0.25 ± 0.02	0.62 ± 0.05[b,c]
0.5	0.3	0.24 ± 0.07	0.60 ± 0.18[b,c]
0.5	1.0	0.17 ± 0.07	0.42 ± 0.17[c]
2.0	0.0	0.30 ± 0.06	3.04 ± 0.56[d]
2.0	0.1	0.21 ± 0.04	2.06 ± 0.37[e]
2.0	0.3	0.12 ± 0.01	1.17 ± 0.15[e,f]
2.0	1.0	0.03 ± 0.00	0.34 ± 0.04[f]
10	0.0	0.19 ± 0.02	9.53 ± 0.73[g]
10	0.1	0.15 ± 0.01	7.67 ± 0.50[h]
10	0.3	0.14 ± 0.02	7.09 ± 0.86[h]
10	1.0	0.08 ± 0.01	3.95 ± 0.30[i]

Within a group at the same level of calcium, means having different superscript letters (b to i) are significantly different at $P < .05$.
[a] Values are means ± SD (n = 3).

4. Discussion

Caco-2 cells are commonly used to study the uptake of nutrients at their apical membranes or transport of nutrients through the cells from the apical to the basolateral membrane. Although transport data clearly model intestinal permeability, uptake data may be confounded by the possibility that the ion or molecule is adsorbed onto the membrane rather than absorbed into the cell. In calcium absorption in the presence of InsP$_6$, calcium phytate appears to bind to the membrane because cells treated with *N*-ethylmaleimide yielded results similar to those from untreated cells. Consequently, Caco-2 cells may not be very useful for studying the effects of inositol phosphates on the uptake of calcium or, possibly, other polyvalent minerals unless a specific biomarker, such as ferritin for iron uptake [19], is measured. Although calcium influx through L-type calcium channels was enhanced by 10 μmol/L of intracellular InsP$_6$ in pancreatic β cells [11] and 20 μmol/L of InsP$_6$ in hippocampal neurons [12], no conclusive indication of a potential enhancement of calcium absorption in the presence of extracellular InsP$_6$ was observed here in Caco-2 cells.

A dose response was observed for the transport of calcium ions through Caco-2 cell layers in the presence of InsP$_6$. Therefore, the Caco-2 cell model may be satisfactory for studying the effects of InsP$_6$ and other inositol phosphates on calcium transport.

Although the fractional inhibition of calcium transport increased with the concentration of InsP$_6$, the total amount of transported calcium still increased with the concentration of calcium from 0.5 to 10 mmol/L. Hence, excess calcium may help to overcome the negative effect of InsP$_6$ that is sometimes observed on the absorption of calcium from foods. For example, both the fractional and total absorption of calcium by adult women from a wheat bran cereal were increased in the presence of milk [20], which contains approximately 25 mmol/L of calcium (101 mg per 100 g) [21].

Kennefick and Cashman [8] measured the transport of 0.5 mmol/L of calcium through Caco-2 cells in the presence of wheat and barley fibers. They found that wheat fiber providing 2.4 mmol/L of phytate decreased the rate of calcium transport by 19%, relative to a cellulose fiber control. Dephytinization of the wheat fiber by phytase eliminated its inhibitory effects on calcium transport, supporting the hypothesis that phytate is the principal inhibitory component of cereal fibers. The ingestion of phytase added to the diet may be an effective means to improve calcium utilization [22].

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